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The antimicrobial activity of honey against common equine wound bacterial isolates

R. Carnwath [a], E.M. Graham [b], K. Reynolds [b], P.J. Pollock [a,*]

[a] Weipers Centre for Equine Welfare, Division of Large Animal Sciences and Public Health, School of Veterinary Medicine, University of Glasgow, 464 Bearsden Road, G61 1QH Glasgow, Scotland, United Kingdom

[b] Division of Veterinary Biosciences, School of Veterinary Medicine, University of Glasgow, 464 Bearsden Road, G61 1QH Glasgow, Scotland, United Kingdom

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ABSTRACT

Delayed healing associated with distal limb wounds is a particular problem in equine clinical practice. Recent studies in human beings and other species have demonstrated the beneficial wound healing properties of honey, and medical grade honey dressings are available commercially in equine practice. Equine clinicians are reported to source other non-medical grade honeys for the same purpose. This study aimed to assess the antimicrobial activity of a number of honey types against common equine wound bacterial pathogens. Twenty-nine honey products were sourced, including gamma-irradiated and non-irradiated commercial medical grade honeys, supermarket honeys, and honeys from local beekeepers. To exclude contaminated honeys from the project, all honeys were cultured aerobically for evidence of bacterial contamination. Aerobic bacteria or fungi were recovered from 18 products. The antimicrobial activity of the remaining 11 products was assessed against 10 wound bacteria, recovered from the wounds of horses, including methicillin resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Eight products were effective against all 10 bacterial isolates at concentrations varying from <2% to 16% (v/v). Overall, the Scottish Heather Honey was the best performing product, and inhibited the growth of all 10 bacterial isolates at concentrations ranging from <2% to 6% (v/v).

Although Manuka has been the most studied honey to date, other sources may have valuable antimicrobial properties. Since some honeys were found to be contaminated with aerobic bacteria or fungi, non-sterile honeys may not be suitable for wound treatment. Further assessment of gamma-irradiated honeys from the best performing honeys would be useful.

Introduction

Substances produced by Honey bees (*Apis mellifera*), including propolis, honey, wax and venom have been used for their medicinal properties throughout history. However, it is the potential wound-healing benefit of honey that has been the primary focus of interest in recent times (Allen et al., 1991). In human beings, research into the therapeutic effect of honey has largely focused on its antimicrobial properties, which are attributed to many factors including acidity, hydrogen peroxide content, osmolarity and phytochemical components (Moore et al., 2001). In addition to inhibiting microbial growth, some of these factors may also have a role to play in controlling inflammation and promoting the healing process through the modulation of cytokines, fibroblast proliferation and angiogenesis (Tonks et al., 2003).

Many varieties of honey are available, differing in constitution and quality between types, and even between batches (French et al., 2005). Some of this variation is due to the type of plant from which the nectar and pollen is collected, the country of origin, and the method of production. The most commonly used medicinal honey is produced by bees foraging Manuka plants (*Leptospermum scoparium*), native to Australia and New Zealand. Manuka honey is believed to have superior antimicrobial properties due to factors other than hydrogen peroxide content. These factors may be due to an as yet poorly understood set of phytochemical properties, and/or to the presence of methylglyoxal, which is derived from dihydroacetone in the nectar of the Manuka flower (Mavric et al., 2008). This non-peroxide property of Manuka honey has been classified as the Unique Manuka Factor (UMF), which is determined by comparison to a standard phenol concentration (Snow and Manley-Harris, 2004).

Equine wounds, particularly those involving the distal portion of the limbs, often undergo prolonged complex healing and may enter a non-healing state with obvious financial and welfare implications. There are many factors which lead to delayed wound healing in horses, and among the most common of these is infection (Hendrickson, 2012). Many chronic equine wounds heal by

* Corresponding author. Tel.: +44 141 330 5999.
E-mail address: patrick.pollock@glasgow.ac.uk (P.J. Pollock).

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second intention and it has been suggested that honey should allow for a better quality of wound repair as it stimulates the initial inflammatory response in leukocytes, increasing the production of cytokines that modulate fibroblast proliferation and angiogenesis (Tonks et al., 2003; Bischofberger et al., 2011).

The protocol for using honey to manage wounds in veterinary practice is highly variable. Some clinicians purchase inexpensive honeys intended for human consumption, and others opt to use standardised, medical grade, gamma-irradiated Manuka honey which in some cases has been incorporated directly into a wound care product or dressing. Since most research studies have been based on medical grade Manuka honey, the properties of other types of honey and bee products are poorly understood.

The aim of this study was to determine the effect of a number of different types, sources and preparations of uncontaminated honey on the growth of common equine wound bacterial pathogens.

Materials and methods

Approval for the project was granted from the Ethics and Welfare Committee at the School of Veterinary Medicine, University of Glasgow.

Honey samples

In total, 28 individual honeys and one commercial sugar solution (Honey Bee Feed) were obtained from a variety of sources ranging from commercial sources of medical grade honey, supermarkets or local beekeepers, and products were refrigerated prior to use (Table 1). Gamma-irradiated and non-irradiated preparations of the same medical grade honey were available and tested separately (Medical Brand 1). All 29 products were cultured aerobically on 5% sheep blood agar and MacConkey agar (E & O Laboratories) overnight at 37 °C; any contaminated honeys were excluded from the second part of the study.

Table 1
Source and culture results of products tested.

	Products tested	Source	Aerobic contamination	
1	Medical brand 1 Manuka honey sterilised (gamma-irradiated)	Manufacturer	No	
2	Medical brand 1 Manuka honey non-sterile (non-irradiated)	Manufacturer	No	
3	Medical brand 2 Manuka honey	Manufacturer	No	
4	Manuka honey 20+	Shop bought	No	
5	Manuka 10+	Shop bought	No	
6	Manuka 5+	Shop bought	Yes	*Bacillus* spp.
7	Heather honey (local)	Bee keeper	No	
8	Heather honey (local)	Shop bought	Yes	*Bacillus* spp.
9	Heather honey	Shop bought	Yes	*Bacillus* spp.
10	Blossom honey	Shop bought	No	
11	Clover honey	Shop bought	Yes	*Bacillus* spp.
12	Orange Blossom honey	Shop bought	Yes	*Bacillus* spp.
13	Lime honey	Shop bought	Yes	*Bacillus* spp.
14	Vipers Bugloss honey	Shop bought	No	
15	Inverness floral (from hive frame)	Bee keeper	No	
16	Inverness floral (from jar)	Bee keeper	Yes	*Bacillus* spp.
17	Glasgow floral (derived from jar)	Bee keeper	No	
18	Supermarket honey 1	Shop bought	Yes	*Bacillus* spp.
19	Supermarket honey 2	Shop bought	Yes	*Bacillus* spp.
20	Supermarket honey 3	Shop bought	Yes	*Bacillus* spp.
21	Supermarket honey 4	Shop bought	Yes	*Enterobacteriaceae* spp.
22	North African Thyme honey	Shop bought	Yes	*Bacillus* spp.
23	North African Coriander honey	Shop bought	Yes	*Bacillus* spp.
24	North African Eucalyptus honey	Shop bought	Yes	*Bacillus* spp.
25	North African Lavender honey	Shop bought	Yes	*Bacillus* spp.
26	North African Ziziphus honey	Shop bought	Yes	*Proteus* spp.
27	North African Euphobia honey	Shop bought	Yes	Fungus
28	Middle Eastern honey	Shop bought	Yes	*Bacillus* spp.
29	Sugar solution (commercial bee winter feed)	Shop bought	No	

Microbe collection and characterisation

The 10 bacterial isolates selected for testing came from a variety of sources, including the wounds of horses presented to the Weipers Centre Equine Hospital at the University of Glasgow; healthy skin samples from a group of horses at livery; equine wounds presented to external veterinary practitioners, and from the University Veterinary Hospital, University College Dublin (Table 2). All samples submitted to the University of Glasgow were cultured aerobically on 5% sheep blood agar and MacConkey agar for 48 h. Isolates were identified by their morphology and analytical profile index (API; BioMérieux,) testing. An antibiogram for each isolate was generated using the disc diffusion method on Diagnostic Sensitivity Test agar (DST; E & O Laboratories); antimicrobial susceptibility discs were sourced from Oxoid. Isolates were stored at −80 °C using a commercial microbead preservation system (Pro-lab Diagnostics).

Preparation of honey-agar solution

The method we used was slightly modified from that described by Cooper et al. (2002). The density of all honeys was assumed to be 1.37 g/mL (Cooper et al., 2002), and only uncontaminated honeys were selected. Double-strength nutrient agar solution was prepared, sterilised and held at 50 °C in a water bath. A 32% (v/v) solution of each honey was prepared in sterile distilled water (dH_20) using aseptic techniques. If required, honeys were dissolved using a sterile magnetised stirrer at 37 °C. Serial dilutions of each honey were prepared in sterile dH_2O at 4% increments (28–4% v/v), and mixed with an equal volume of double-strength nutrient agar. A final volume of 20 mL was poured into each of 3×90 mm labelled Petri dishes, which were left to dry. The final concentrations of honey used in the study were 16%, 14%, 12%, 10%, 8%, 6%, 4% and 2% (v/v). The final concentrations of sugar solution used were 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10% and 5% (v/v).

Preparation and inoculation of bacterial isolates

Ten bacterial isolates were used in this study; the origin and antibiogram of each isolate is shown in Table 2. To prepare samples for inoculation, each isolate was recovered from a microbead, streaked onto nutrient agar plates and incubated aerobically at 37 °C for 18–24 h. For each isolate, between three and five colonies were selected and transferred into a sterile glass-capped tube containing dH_20 and vortexed. The turbidity was adjusted to a 0.5 McFarland standard (BioMérieux; 1.5×10^8 cfu/mL), and confirmed using a colorimeter (Viek). A suspension of each isolate was prepared (1.5×10^7 cfu/mL) and used within 30 min. The honey-agar plates were inoculated in duplicate with 10 isolates in 1 μL volumes each containing 1.5×10^4 cfu organisms (Denley Multipoint Inoculator A400), and incubated aerobically overnight at 37 °C. An uninoculated plate at each honey concentration was used as a negative control to detect contamination. Three single-strength nutrient plates were inoculated with all 10 isolates as a positive growth control.

Interpretation of results

The plates were examined after 16–24 h culture, and the presence or absence of visible colony formation was recorded for each isolate at each honey concentration. The honey minimum inhibitory concentration (MIC) value was recorded as the lowest concentration of honey at which bacterial growth was absent. MIC values were obtained for each honey against each bacterial isolate. If an isolate was inhibited at the lowest concentration (2%), the MIC was recorded as <2%. Growth at the highest concentration tested i.e. 16%, meant that the MIC was recorded as >16%. The MIC values reported are the mean of four replicates except Heather and Inverness Floral honeys (two replicates).

Results

Bacterial contamination

Of the 29 products tested, 18 were contaminated with pure cultures of aerobic bacteria or fungi (Table 1). *Bacillus* spp. were recovered from 15 products, *Proteus* spp. was recovered from a single supermarket honey, an unidentified *Enterobacteriaceae* organism was recovered from a commercial North African honey, and an unidentified fungus was recovered from a second commercial North African honey.

Table 2
Source and antibiograms of 10 selected bacterial isolates.

Bacterial isolate	Source	Antibiogram									
		Enr	GN	OTC	P	C	TMS	AK	CEF	MNZ	CMP
MRSA	Equine metacarpal wound, GU	S	I	R	R	R	I	I	R	R	S
Staphylococcus aureus	Equine isolate, UCD	S	R	S	R	S	S	R	I	R	I
Escherichia coli	Equine withers wound, external practice	R	R	R	R	S	R	S	S	R	S
Streptococcus equi	Equine isolate, GU	S	R	S	S	S	R	R	S	R	S
S. equi subsp. *zooepidemicus*	Equine isolate, GU	R	R	R	I	S	S	S	S	S	R
Enterococcus faecalis	Equine forelimb wound, external practice	R	R	S	S	R	R	R	R	R	R
Acinetobacter baumannii	Equine isolate, UCD	I	R	R	R	R	R	I	R	R	R
MRSE	Equine isolate, GU	R	R	R	R	I	R	S	I	R	I
Staphylococcus sciuri	Livery horse, GU	S	R	S	S	R	R	S	S	R	S
Pseudomonas aeruginosa	Canine isolate, GU	R	I	R	R	R	R	I	R	R	R

MRSA, methicillin-resistant *Staphylococcus aureus*; MRSE, methicillin-resistant *Staphylococcus epidermidis*; GU, School of Veterinary Medicine, University of Glasgow; UCD, University College Dublin; Enr, enrofloxacin; GN, gentamycin; OTC, oxytetracycline; P, penicillin G; C, ceftiofur; TMS, trimethoprim sulphamethoxazole; AK, amikacin; CEF, cefquinome; MNZ, metronidazole; CMP, chloramphenicol; S, sensitive; I, intermediate; R, resistant.

Minimum inhibitory concentration

The antibacterial properties of the 11 uncontaminated products were evaluated against the 10 selected bacterial isolates, and the results are summarised in Table 3. Eight of the 11 products were effective against all 10 bacterial isolates at concentrations ranging from <2% to 16% (v/v). Overall, the Scottish Heather Honey was the best performing product, and inhibited the growth of all 10 isolates at concentrations ranging from <2% to 6% (v/v). The sugar solution was the worst performing product, failing to inhibit growth of 5/10 isolates at concentrations of 45% and below. Brand one medical grade honey was tested in a sterile, gamma-irradiated form and in a non-irradiated form. There was no difference in MIC values between the irradiated and non-irradiated preparations.

Although *Acinetobacter baumanii* and *Pseudomonas aeruginosa* appeared to be the most resistant isolates (Table 2), all 10 honeys were able to inhibit growth of these isolates at concentrations as low as 4%. The isolate most resistant to the honeys appeared to be *Enterococcus faecalis*, although 8/10 honeys were able to inhibit growth at concentrations ranging from 6% to 16% (v/v).

Discussion

The results of this study demonstrate that certain varieties and sources of honey are effective at inhibiting microbial growth in vitro at very low concentrations. These effective honeys include medical grade Manuka honey used in commercial wound products and dressings, Manuka honey (greater than UMF 10), and also include some commercially available monoculture honey from local suppliers, and locally produced honey. In addition our study suggests that the use of certain non-medical grade honeys, derived from mixed floral sources, may not be appropriate for wound care due to the presence of contaminating aerobic bacteria.

Prior to the study, all honeys were aerobically cultured to rule out the presence of aerobic bacteria that could interfere with the antimicrobial assessment. The presence of anaerobic bacteria, non-cultivable bacteria, or fungi, cannot be excluded as appropriate testing was not done. Indeed, the presence of *Clostridium botulinum* spores in commercial honey sources is an established although rare public health hazard (Smith et al., 2010). Most *Bacillus* species are saprophytes that are widely distributed in the environment with no pathogenic potential (Quinn et al., 2002), however in addition to the known wound pathogen *Bacillus cereus*, some other *Bacillus* species are suspected to be opportunistic wound pathogens (Logan, 1988). As a result, *Bacillus*-contaminated honeys are not recommended for therapeutic use.

Potentially pathogenic organisms, the *Enterobacteriaceae* and *Proteus* isolates, were cultured from two honey samples and present a public health concern. The source of the two Gram-negative non-sporing bacterial contaminants recovered in this study is unknown, although these are most likely to have been introduced during the manufacturing process. It is possible that these originated from the authors' laboratory, but this seems unlikely since all products were handled under identical aseptic conditions and multiple batches of the same honey gave similar results.

In this study, the unprocessed Inverness Floral honey was found to be uncontaminated whereas the processed honey from the same source was contaminated with *Bacillus* spp. Since aerobic bacteria were cultured from many of these honeys, and the presence of other microorganisms cannot be excluded, only sterilised honey

Table 3
Mean MIC and standard deviation of each tested product against 10 bacterial isolates.

Isolate	Brand 1		Brand 2	Manuka 20+	Manuka 10+	Blossom	Vipers Bugloss	Floral-Glasgow	Heather	Floral-Inverness	Sugar solution
	Sterile	Non-sterile									
MRSA	4 ± 1.2	4 ± 1.2	10 ± 0	3 ± 1	10 ± 0	4 ± 1.4	>16 ± 0	14 ± 1.9	<2 ± 0	4 ± 0	>45 ± 0
S. aureus	6 ± 0	4 ± 1	10 ± 0	2 ± 0	10 ± 0	4 ± 0	16 ± 0.7	14 ± 3.5	<2 ± 0	4 ± 0	>45 ± 0
E. coli	6 ± 1.2	6 ± 0	10 ± 0	4 ± 1.2	12 ± 0	8 ± 0	16 ± 0	>16 ± 0	6 ± 1.4	8 ± 1.4	45 ± 1
S. equi subsp. *equi*	6 ± 0	4 ± 1	10 ± 0	4 ± 1	8 ± 0	4 ± 0	4 ± 0	10 ± 6.4	4 ± 0	6 ± 0	25 ± 0
S. equi subsp. *zooepidemicus*	6 ± 3.5	4 ± 1.2	10 ± 0	4 ± 0	10 ± 0	4 ± 0	6 ± 0	12 ± 6.9	<2 ± 0	6 ± 1.4	30 ± 0
E. faecalis	8 ± 0	8 ± 0	12 ± 0	6 ± 1.2	10 ± 0	16 ± 0	>16 ± 0	>16 ± 0	6 ± 0	8 ± 0	>45 ± 0
A. baumannii	6 ± 1.2	6 ± 1.2	10 ± 0	4 ± 1	12 ± 0	6 ± 0	14 ± 1.4	14 ± 3.5	6 ± 0	10 ± 0	40 ± 1.2
MRSE	4 ± 1.2	6 ± 0	10 ± 0	4 ± 1.2	6 ± 0	4 ± 0	4 ± 0	8 ± 0	4 ± 1.4	8 ± 0	>45 ± 0
S. sciuri	4 ± 1.2	4 ± 0	10 ± 0	4 ± 0	6 ± 0	4 ± 0	8 ± 0	8 ± 4.1	<2 ± 0	6 ± 0	>45 ± 0
P. aeruginosa	8 ± 0	10 ± 0	10 ± 0	8 ± 0	10 ± 0	4 ± 0	12 ± 0	12 ± 5.8	4 ± 0	10 ± 0	30 ± 0

MRSA, methicillin-resistant *Staphylococcus aureus*; MRSE, methicillin-resistant *Staphylococcus epidermidis.*

should be used for incorporation into wound dressings. We found no difference between the ability of medical grade gamma-irradiated honey and non-irradiated honey from the same source to inhibit the bacterial isolates; previous workers have noted that gamma-irradiation has no significant effect on the antibacterial properties of honey, whereas heat treatment for sterilisation does (Mollan and Allen, 1996). The effects of gamma irradiation on the other properties of honey, such as those that promote wound healing, are unknown.

With antimicrobial resistance on the rise and the emergence of multi-resistant bacteria in both human and veterinary medicine, honey offers an exciting addition to conventional antimicrobial drugs. Many yeasts and spore-forming bacteria are inherently resistant to honey, and are frequently recovered from non-sterilised honey. In contrast, most Gram negative non-sporing isolates will not survive in honey for more than a few days. Nonetheless, this is dependent on the bacterium, antimicrobial properties of the individual honey, and the conditions of storage (Snowdon and Cliver, 1995). Resistance acquired in vivo has not yet been reported, and the Gram-negative non-sporing bacteria recovered in this study are unlikely to be honey-resistant. Nonetheless, a recent in vitro study demonstrated that suboptimal concentrations of Manuka honey can allow resistant bacterial wound pathogens to emerge (Cooper et al., 2010). This finding highlights the need to maintain adequate concentrations of an efficacious honey at the wound-dressing interface.

To date, there have been no reports of acquired antimicrobial resistance to honey; this is probably due to multiple synergistic components that give honey its antimicrobial properties. Although the nature of these properties remains to be fully elucidated, several factors are thought to play a part, including osmolarity. However, the comparison of the honeys with the sugar solution (commercial bee feed) in this study suggests that osmolarity alone does not fully explain the antimicrobial activity of honey. The sugar solution failed to inhibit the growth of five isolates even at high concentrations (at least 45%). Hydrogen peroxide content also plays a role although studies investigating the properties of honey have shown several sources to have non-peroxide activity by using catalase to denature the hydrogen peroxide in the honey. Manuka, Clover and Vipers Bugloss are some of the honeys shown to have such non-peroxide activity (Allen et al., 1991). In the present study both Manuka and Vipers Bugloss honeys were tested and although Manuka generally had very effective antimicrobial properties, Vipers Bugloss did not. This suggests that the antimicrobial properties of honey cannot be attributed to non-peroxide levels alone.

It is known that bees do not fly further than approximately 6 km from the hive to collect their nectar source (Hagler et al., 2007), therefore positioning bee hives in the centre of a mono-floral area, for example heather, with a minimum radius of 6 km results in a monofloral heather honey. In our study, the best performing honeys were mono-floral; however, several factors may influence this such as the quality of the honey, as determined by cost, source or UMF, and the botanical sources of the mixed floral honey. The botanical sources can be determined by Fourier-transformed infrared spectroscopy analysis, amongst other methods (Etzold and Lichtenberg-Kraag, 2008).

Manuka honey is currently the only source of medical grade honey available in dressings and wound treatment products. Our study shows that many sources of honey have antimicrobial properties, and may therefore also be effective in the treatment of wound infections. Consequently, it may prove unnecessary to transport Manuka honey from New Zealand when more local sources may be as, or even more, effective. In many regions of the world access to expensive antimicrobial drugs is limited; therefore, locally sourced honey may provide an inexpensive alternative. The effect of gamma irradiation on the antimicrobial properties and wound promoting activity of the most promising honeys in this study should be assessed.

Most tested honeys were effective at concentrations of 16% or less, much lower than is likely to occur at the surface of an infected wound treated with honey, even taking into account dilution by wound fluid and exudate. Further work is required to determine the effect of honey on microbial growth in vivo. In the future it may be possible to identify honeys with activity against specific bacteria. This would allow the clinician to select the most appropriate honey type for the infecting organism.

Conclusions

This report outlines the effect of a number of commercially available honeys against ten commonly encountered equine wound microbial pathogens. In contrast to previous reports, a number of the honey samples in this project were even more effective at inhibiting microbial growth than Manuka honey and as such are potentially of interest for the treatment of infected wounds in horses and other species. A number of the commercially available honey samples tested were contaminated with potentially pathogenic organisms and as such clinicians should be cautious about the use of non medical grade honey in wound therapy. Further work is indicated to elucidate the mechanism of action of these products.

Conflict of interest statement

None of the authors have any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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